Available online at www.sciencedirect.com
ScienceDirect
Comparative Biochemistry and Physiology, Part B 149 (2008) 517–523
CBP
www.elsevier.com/locate/cbpb

Molecular characterization and responsive expression of a defender against apoptotic cell death homologue from the Hessian fly, *Mayetiola destructor*

Omprakash Mittapalli [a,*,1], Richard H. Shukle [b]

[a] *Max-Planck Institute for Chemical Ecology, Department of Molecular Ecology, Jena, Germany*
[b] *USDA-ARS, Department of Entomology, Purdue University, West Lafayette, IN 47907 USA*

Received 8 October 2007; received in revised form 5 December 2007; accepted 5 December 2007
Available online 8 December 2007

Abstract

Apoptosis or programmed cell death is an active process occurring in multicellular organisms to maintain growth and development. The Hessian fly, *Mayetiola destructor*, is rapidly emerging as a model insect species to study insect-plant interactions and to decipher some exceptional physiological phenomena. In this study, we report the characterization and expression profiles of a putative Hessian fly defender against apoptotic cell death (DAD1) homologue designated *MdesDAD1*. The deduced amino acid sequence of MdesDAD1 revealed significant similarity (75% identity, 9e-42) to other insect and non-insect DAD1 sequences. Phylogenetic analysis grouped MdesDAD1 within a sub-clade consisting of other insect DAD1 homologues. Quantitative analysis indicated constitutive levels of *MdesDAD1* mRNA in all the tissues examined but an altered expression pattern during development, wherein the highest mRNA levels observed were prior to pupation. Most interestingly, *MdesDAD1* transcript was found to be up-regulated during incompatible (larvae reared on resistant wheat) Hessian fly/wheat interactions compared to compatible (larvae reared on susceptible wheat) interactions. These results suggest MdesDAD1 to have a putative role in the inhibition of unwanted apoptosis triggered during development and in incompatible Hessian fly/wheat interactions. The results obtained provide clues to plausible insect and host-plant factors that could be responsible for the induction of *MdesDAD1*.

Keywords: Defender against apoptotic death (DAD1); Hessian fly; mRNA expression

1. Introduction

Apoptosis or programmed cell death (PCD) is a universal phenomenon occurring in eukaryotes including mammals, insects and plants. Characteristic features of apoptosis include down-regulation of anti-apoptotic genes such as DAD1 (defender against apoptotic cell death, Yamada et al., 2004), DNA fragmentation, nuclear condensation and cellular shrinkage (Steller, 1995; Higuchi, 2003). The first putative DAD gene was reported from a mutant (temperature-sensitive) hamster cell-line, tsBN7 (Nakashima et al., 1993).

The response of DAD1 genes to inhibit PCD has been well studied in an array of organisms. Specifically, the up-regulation of these homologues in *Araneus ventricosus* and *Argopecten irradians* are thought to defend against unwanted cell death upon exposure to external stimulus such as temperature (Lee et al., 2003; Zhu et al., 2007). The induction of DAD1 genes have also been observed during development in *Caenorhabditis elegans* (Sugimoto et al., 1995) and *Bombyx mori* (Tsusuki et al., 2001). On the contrary, down-regulation of a DAD1 gene was observed in flower petals during the senescence phase (Orzaez and Granell, 1997). These studies clearly indicate the response of DAD1 genes attributed toward external stimuli but scarce knowledge exists in insects on their characterization and expression profiles in response to similar and/or other external stimuli.

The Hessian fly (*Mayetiola destructor*) is an important agricultural insect pest of wheat worldwide (Shukle, 2003). More recently, it is emerging as a model system to decipher

* Corresponding author. Max-Planck Institute for Chemical Ecology, Department of Molecular Ecology, Hans Knoell Str. 8, Jena D-07745, Germany. Tel.: +49 3641 57 1121; fax: +49 3641 57 1102.
E-mail address: omittapalli@ice.mpg.de (O. Mittapalli).
[1] Previous address: Purdue University, Department of Entomology, West Lafayette, IN 47907 USA.

1096-4959/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2007.12.001

galling interactions at the molecular level and is revealing some exceptional physiological phenomena (Mittapalli et al., 2006, 2007b). Hessian fly/wheat interactions can be classified primarily into two types: compatible interactions, wherein 1st-instar larvae are successful in establishing a sustained feeding site and subsequently complete their development on susceptible wheat seedlings; incompatible interactions, wherein 1st-instar larvae are deprived of sustained feeding on resistant wheat and usually die within 5–6 days post-hatch (Painter, 1930; Shukle et al., 1992).

To date there have been no reports on DAD1-like genes in the Hessian fly. Furthermore, it is yet to be revealed whether Hessian fly larvae feeding on host wheat plants triggers apoptosis (leading to their death) or not. Thus, the expression of a putative Hessian fly DAD1 could represent a valuable biomarker for apoptotic cell death elicited by resistant plant factors. We hypothesize that larvae feeding on resistant wheat plants encounter greater stress-induced challenges when compared to larvae feeding on susceptible wheat plants and therefore up-regulate the expression of anti-apoptotic genes. In this paper, we present data on gene characterization and mRNA expression patterns of a Hessian fly DAD1 gene (designated as *MdesDAD1*) that was recovered from a larval midgut expressed sequence tag (EST) project. The high levels of *MdesDAD1* mRNA observed in larvae during development and in incompatible interactions suggest that *MdesDAD1* responds to unwanted developmentally-regulated and stress-induced PCD. Results are discussed in the context of the plausible sources responsible for an early onset of PCD in the Hessian fly.

2. Materials and methods

2.1. Experimental insect and plant material

A laboratory culture of the Hessian fly Biotype L was used in this study. This biotype is defined as virulent (able to survive and stunt) on wheat (*Triticum aestivum*) lines carrying resistance genes *H3*, *H5*, *H6* and *H7H8* but avirulent (unable to survive and stunt) on the wheat line 'Iris' that carries the resistance gene H9. Biotype L was collected from a filed collection made from Posey County, Indiana in 1986 and maintained as described by Sosa and Gallun (1973). The other wheat line used in this study was 'Newton', which carries no gene for resistance and is near isogenic to Iris. Mass-infestations were done in chambers with Biotype L adults on Newton seedlings that were at the one-leaf stage. Hatched larvae were dissected under a stereoscopic microscope using forceps. Larvae were collected in 1.5 mL microcentrifuge tubes and were either used immediately or flash-frozen in liquid nitrogen and subsequently stored at −80 °C for later use.

2.2. Larval tissue dissection and RNA extraction

Approximately 300 late 1st- and early 2nd-instar larvae (Biotype L on Newton) were dissected immersed in ice-cold Schneider's insect medium (Sigma-Aldrich, St. Louis, MO, USA). Midguts, salivary glands, fat bodies and Malphigian tubules were isolated as described by Mittapalli et al. (2005a). All the tissues were collected in 100 mL of ice-cold Schneider's contained in a 1.5 mL micro-centrifuge tube. Collected tissues were flash frozen in liquid nitrogen immediately following dissection and stored at −80 °C until RNA was isolated. Total RNA from tissues, developmental stages (1st-, 2nd-, 3rd-instars, pupae and adults) was isolated using the RNAqueous-4PCR kit from Ambion (Austin, TX, USA). For performing interaction studies, total RNA was isolated (as described above) from larvae reared on susceptible Newton plants (representing compatible interactions) and on resistant Iris plants (representing incompatible interactions) 6 through 96 h post-hatch. Additionally, larvae were also reared on moist filter paper for 24 h as a control. Larvae on moist filter paper were included only up to 24 h post-hatch due to their inability to survive for longer periods.

2.3. Construction of midgut-specific cDNA libraries

An un-normalized cDNA library was constructed from RNA extracted from the midgut tissue using a SMART™ cDNA library construction kit from BD Biosciences (Palo Alto, CA, USA). The manufacturer's protocol was followed with one modification: instead of performing the cloning of the PCR fragments into the phage vector supplied with the kit, the fragments were cloned directly into the PCR4-TOPO vector included in a TOPO TA cloning for sequencing kit from Invitrogen (Carlsbad, CA, USA). Plasmid DNA was isolated using a Qiagen BioRobot 3000 and subsequent high throughput sequencing was performed in an ABI 3700 DNA analyzer by the Purdue Genomics Center. The cloned EST fragments were sequenced using a primer designed to the 5′ cloning oligonucleotide of the vector from the cDNA library construction kit.

2.4. Sequence annotation and multiple alignment

Sequence similarity searches and annotations were done using the basic local alignment search tool (BLAST) programs (Altschul et al., 1990) on the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) website (http://www.ncbi.nlm.nih.gov/). The protein secondary structure of MdesDAD1 was predicted using the PSIPRED protein structure prediction server at http://bioinf.cs.ucl.ac.uk/psipred/ (Jones, 1999). Multiple alignment of the deduced amino acid sequence of MdesDAD1 with other DAD1 sequences was performed using ClustalW (http://align.genome.jp/).

2.5. Phylogenetic analysis

In order to reveal the relationship of the Hessian fly DAD1 with similar genes from other organisms, a phylogenetic tree was constructed with the amino acid sequences of DAD1 from arthropods and mammals. A DAD1 from *Caenorhabditis elegans* was used as the out-group. The peptide sequences were aligned with the ClustalX program, version 8.1 with 11 updates (Thompson et al., 1997). The phylogenetic tree was calculated by the distance/neighbor-joining method in the ClustalX program. Bootstrap values for the branches were obtained with 1000 replications.

2.6. Quantitative analysis of the Hessian fly DAD1 mRNA

Levels of mRNA encoding the Hessian fly DAD1 in the larval tissues examined were measured via quantitative real-time PCR (qRT–PCR). qRT–PCR was also used to assess transcript levels of *MdesDAD1* during larval development on susceptible plants (compatible interaction) and on resistant plants (incompatible interaction). The primer sequences used in this study (Table 1) were designed using the software Primer Express from Applied Biosystems (Foster City, CA, USA). First strand cDNA synthesis was done as mentioned by Mittapalli et al. (2007a) using the SuperScript First Strand cDNA Synthesis kit from Invitrogen. Subsequent quantification of the obtained cDNAs was also done as described by Mittapalli et al. (2007a), which was based on the Relative Standard Curve method (User Bulletin #2: ABI Prism 7700 Sequence Detection System http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf) that uses serial dilutions of a cDNA sample containing the target sequence. The parameters for PCR cycling included 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The entire analysis was performed using a Hessian fly ubiquitin as an internal standard, which has shown constant expression in the Hessian fly (Mittapalli et al., 2007a). Ubiquitin has been used as a suitable internal reference in a number of other experimental systems (Jin et al., 2003; Yuan et al., 2006).

2.7. Statistical analysis

The statistical model used for determining the expression levels of *MdesDAD1* in tissues and in the developmental stages assayed included treatment and interaction between treatments. For the analysis of expression during different Hessian fly/wheat interactions (compatible and incompatible), the statistical model included treatment, time points, and interaction between treatments and time points as fixed effects. For calculations of significance, the logs of the derived relative expression values (REVs) for each gene (*MdesDAD1* and *MdesUB*) were analyzed by Analysis of Variance (ANOVA) using the PROC MIXED procedure of SAS (SAS Institute Inc. SAS/STAT User's Guide, Version 9.1). Treatment differences at each time point were evaluated using orthogonal contrasts and considered statistically significant if the *P*-value associated with the contrast was <0.05. Two biological replicates were included for the tissue/developmental expression analysis, whereas, three biological replicates were used for expression analysis of larvae on susceptible plants versus larvae on resistant plants. For each biological replicate a pool of individuals/tissues (~100) represented one sample. Biological replicates were included as a random effect in the analysis model. Further, two technical replicates were included in each biological replicate. Technical replicates were included to correct for errors during pipetting within each biological replicate. The standard error represented the variance in these biological replicates for the respective analysis.

Relative fold increase in the case of tissue expression was calculated by taking the salivary gland mRNA sample the calibrator as it showed the least level of *MdesDAD1* mRNA (confer User Bulletin #2: ABI Prism 7700 Sequence Detection System vide supra). The fold-change of the Hessian fly DAD1 transcript in the midgut, fat bodies and Malphigian tubules were calculated relative to the salivary glands. Similarly, the expression in the adults was taken as the calibrator during development. The fold increase in expression of *MdesDAD1* in the interaction experiments was determined by dividing the REV for larvae on resistant plants (incompatible interactions) by the REV for larvae on susceptible plants (compatible interactions) for all the time points examined (6–96 h post-hatch). Fold change in gene expression of *MdesDAD1* in larvae on moist filter paper was calculated by dividing the REV for larvae on moist filter paper by the REV for larvae during compatible interactions.

Table 1
Oligonucleotide sequences used for quantifying the transcripts of *MdesDAD1* and *MdesUB*

Name of primer	Primer sequence (5′–3′)	Tm (°C)	Position in cDNA
Target gene			
MdesDAD1-F	ATCGACCATCAGTTGTTTTGTTCTT	58.1	180-204
MdesDAD1-R	CATAACCTCGTTCCGGTGAAA	57.9	260-280
Internal control			
MdesUB-1F	CCCCTGCGAAAATTGATGA	54.5	109-127
MdesUB-1R	AACCGGACTACTTGCATCGAA	56.7	153-173

3. Results

3.1. Characterization of the Hessian fly DAD1 homologue

A full length cDNA coding for a putative DAD1 homologue from the Hessian fly consisted of 339 bp and had an open reading frame of 113 amino acids (Fig. 1A). The recovered sequence had a 5′-untranslated region (UTR) of 75 bp and a 3′-UTR of 181 bp. The 3′-UTR included the consensus polyadenylation signal sequence AATAAA, which matches with other species (Zhu et al., 2007). Three closely spaced transmembrane domains spanned the gene sequence. Both the DNA and deduced amino acid sequences were submitted to GenBank and assigned the accession number EU188857. This gene has been designated by the authors as '*MdesDAD1*'. The deduced amino acid sequence of MdesDAD1 showed greatest similarity (75% identity, 9e-42) to that of a mosquito DAD1 (*Anopheles gambiae*, XP_316953). The predicted secondary structure (Jones, 1999) obtained for MdesDAD1 revealed the presence of four large alpha-helices and five small intermittent coiled regions but no beta-strands across the entire deduced protein sequence (Fig. 1B). Except for the first transmembrane domain, the other two domains were represented by only alpha-helices.

A multiple sequence alignment of MdesDAD1 with other insect and non-insect DAD1 protein sequences revealed several homologous regions (Fig. 2). The obtained alignment indicated 40 amino acid residues (35%) that were identical amongst all the taxa included in the analysis. These conserved residues were spread across all the three transmembrane domains but were observed in greater

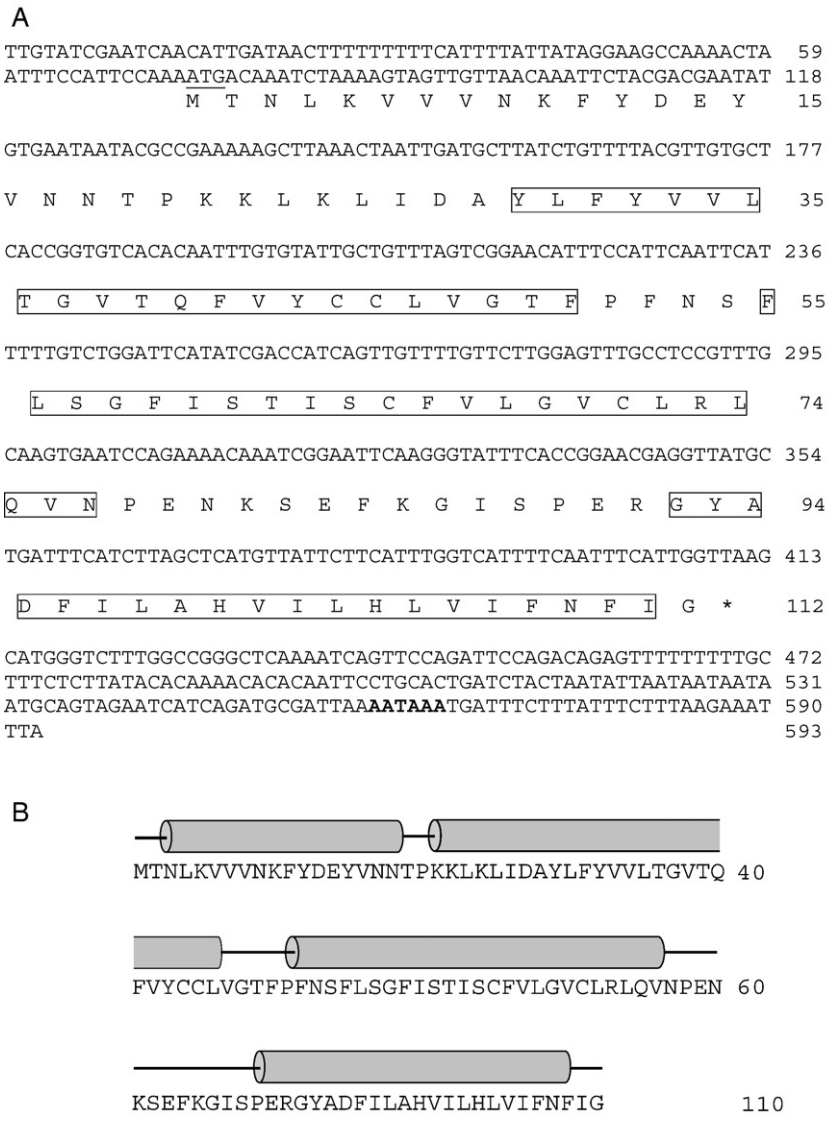

Fig. 1. Characterization of the Hessian fly DAD1, *MdesDAD1*. (A) Nucleotide and deduced amino acid sequences of MdesDAD1. The start codon (ATG) is underlined and the stop codon (TAA) is shown as an asterisk. An AATAAA sequence matching the consensus polyadenylation signal in other species is bolded. The three closely spaced transmembrane domains are boxed. This cDNA has been deposited in GenBank bearing the accession number EU188857. (B) Predicted secondary structure of MdesDAD1. The top of the figure shows the predicted protein components corresponding to the lower deduced amino acid sequence. Cylinders and solid black lines represent alpha-helices and coils, respectively.

number towards the C-end of the MdesDAD1 deduced protein sequence.

3.2. Phylogenetic analysis

A distance/neighbor-joining phylogenetic tree was constructed to reveal the relationship of MdesDAD1 with other insect and non-insect DAD1 homologues using a *Caenorhabditis elegans* DAD1 as the out-group (Fig. 3). The tree revealed two major clades: (1) arthropod DAD1 homologues including a spider DAD1 (*Araneus ventricosus*) and several dipteran DAD1 homologues; (2) mammalian DAD1 homologues including the taxa *Homo sapiens*, *Mus musculus* etc. The Hessian fly DAD1 (MdesDAD1) grouped within the first major clade. Specifically, MdesDAD1 grouped between other dipteran taxa i.e. *Drosophila melanogaster* and *Ae. aegypti* (Fig. 3).

3.3. Expression profiles of MdesDAD1 mRNA in larval tissues

The transcript levels of *MdesDAD1* was assayed in different larval tissues (midgut, salivary glands, fat bodies and Malphigian tubules) using qRT–PCR. The relative expression of *MdesDAD1* was observed to be highest in Malphigian tubules and least in salivary glands. Thus, the basal expression level in the salivary glands was used to calculate the fold difference in other tissues. However, upon determining the fold increase it was found that there was no significant ($P>0.05$) differential expression (fold changes ≤ 1.5) revealed among the

```
M. destructor    MTN-LKVVVNKFYDEYVNN---TPKKLKLIDAYLFYVVLTGVTQFVYCCLVGTFPFNSFL 56
Ae.aegypti       MTN-IKTVLVKFYDDYTVN---TPKKLKIVDAYLLYILLTGITQFVYCCLVGTFPFNSFL 56
H.sapiens        MSASVVSVISRFLEEYLSS---TPQRLKLLDAYLLYILLTGALQFGYCLLVGTFPFNSFL 57
C.elegans        MAAQVVPVLSKLFDDYQKT---TSSKLKIIDAYMTYILFTGIFQFIYCLLVGTFPFNSFL 57
A.thaliana       MVKSTSKDAQDLFRSLRSAYSATPTNLKIIDLYVVFAVFTALIQVVYMALVGSFPFNSFL 60
                 *          :  .       *. .**::* *: : ::*.  *. *  ***:*******

M. destructor    SGFISTISCFVLGVCLRLQVNPENKSEFKGISPERGYADFILAHVILHLVIFNFIG    112
Ae.aegypti       AGFICTVSCFVLGVCLRLQSNPQNKSQFLGISPERGFADFVFAHVILHLVVVNFIG    112
H.sapiens        SGFISCVGSFILAVCLRIQINPQNKADFQGISPERAFADFLFASTILHLVVMNFVG    113
C.elegans        SGFISTVTSFVLASCLRMQVNQENRSEFTAVSTERAFADFIFANLILHLVVVNFLG    113
A.thaliana       SGVLSCIGTAVLAVCLRIQVNKENK-EFKDLAPERAFADFVLCNLVLHLVIINFLG    115
                 :*.:. :   :*. ***:* * :*: :*  ::.**.:***::.  :****:.**:*
```

Fig. 2. Multiple sequence alignment of MdesDAD1. Shown is the homology of the deduced amino acid sequence of the Hessian fly DAD1 with sequences from *Aedes aegyptyi* (ABF18074), *Homo sapiens* (AH005230), *Caenorhabditis elegans* (AAB96727), and *Arabidopsis thaliana* (NP_174500). Identical residues among all taxa are indicated by "*", conserved substitutions by ":" and semi-conserved substitutions by "." symbols.

tissues examined (data not shown). A 1.05-, 1.02-, and 1.20-fold change were calculated between midgut/salivary glands, fat bodies/salivary glands and Malphigian tubules/salivary glands, respectively.

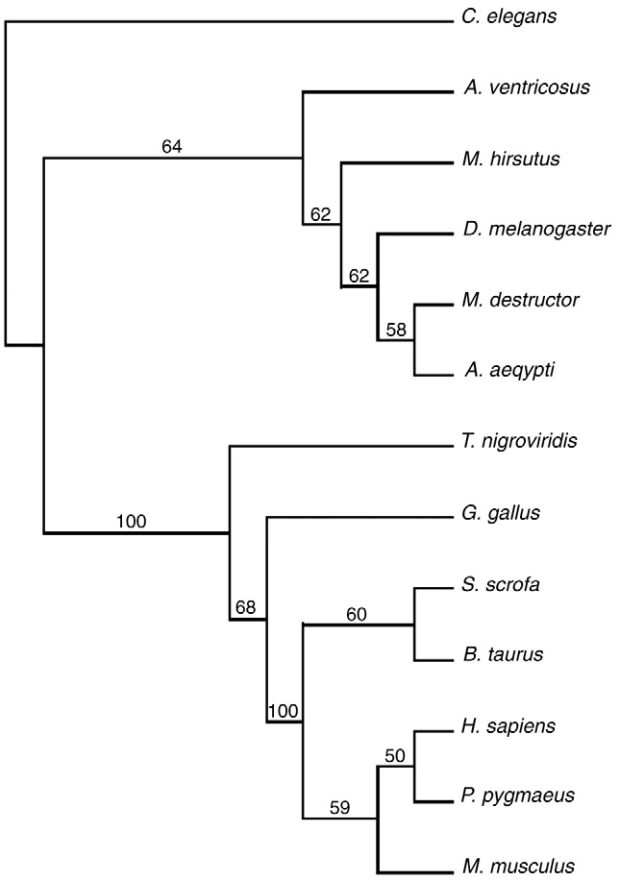

Fig. 3. A phylogenetic tree based on the amino acid sequence for MdesDAD1 and DAD1 sequences from other taxa. A *Caenorhabditis elegans* DAD1 (AAB96727) was included as the outgroup. The distance/neighbor-joining method was used to determine the topology and branch lengths of the phylogram. The percentage of 1000 bootstrap replications supporting each node is shown. Taxa and GenBank accession numbers included are as follows: *Araneus ventricosus*, AAN86571; *Maconellicoccus hirsutus*, ABM55607; *Drosophila melanogaster*, NP_609222; *Mayetiola destructor*, EU188857; *Aedes aegypti*, ABF18074; *Tetradon nigroviridis*, CAF97730; *Gallus gallus*, NP_001007474; *Sus scrofa*, NP_999109; *Bos taurus*, NP_001029933; *Homo sapiens*, AH005230; *Pongo pygmaeus*, Q5RBB4; *Mus musculus*, AAC53098.

3.4. Expression patterns of MdesDAD1 during development

Quantification of *MdesDAD1* mRNA in different developmental stages (including 1st-, 2nd-, and 3rd-larval instars, pupa and adults) was also assessed using qRT–PCR. A high level of *MdesDAD1* expression was observed from late 2nd-instar to mid 3rd-instar larvae (Fig. 4). The peak expression of *MdesDAD1*in late 3rd-instar was followed by an abrupt decrease in the later developmental stages. The lowest level of expression was determined to be in adults. Thus, this basal level of *MdesDAD1* expression in adults was used to determine the fold changes between the developmental stages examined. An average fold increase of 2.8, 4.1, 7.4, and 1.5 were calculated between 1st-instar/adults, 2nd-instars/adults, 3rd-instars/adults and pupae/adults, respectively.

3.5. Differential expression patterns of MdesDAD1 during interactions with wheat

The transcript level of *MdesDAD1* was assessed in larvae infesting both susceptible and resistant wheat, which represent compatible and incompatible Hessian fly/wheat interactions. In addition, larvae were reared on moist filter paper and included as a control. Since larvae during incompatible interactions survive for 5–6 days post-hatch, larval samples were collected

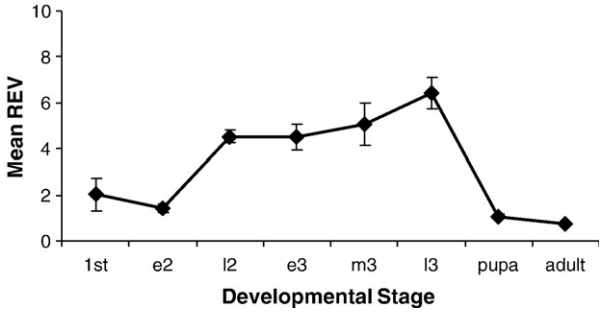

Fig. 4. Expression profile of *MdesDAD1* during development. Quantitative analysis of *MdesDAD1* mRNA in different stages of the Hessian fly development including 1st-instar larvae (1st), early 2nd-instar larvae (e2), late 2nd-instar larvae (l2), early 3rd-instar larvae (e3), mid 3rd-instar larvae (m3) and late 3rd-instar larvae (l3), pupae and adults. Log of the mean relative expression value (REV) is plotted against each of the developmental stage examined. The error bars represent the pooled standard error for two biological replicates. Each biological replicate was represented by two technical replicates.

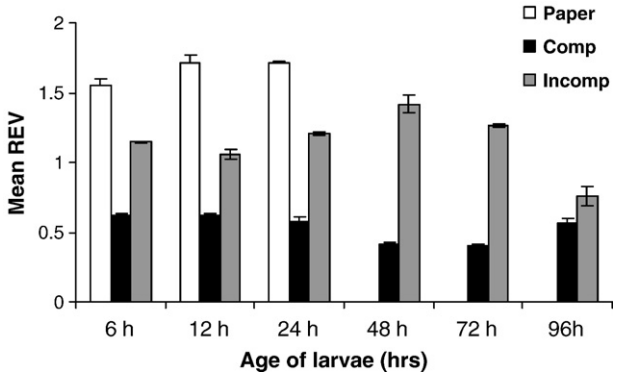

Fig. 5. Temporal expression patterns of *MdesDAD1* during Hessian fly/wheat interactions. Samples of Hessian fly larvae reared on moist filter paper, susceptible wheat (compatible interaction) and on resistant wheat (incompatible interaction) were collected through the 1st-instar (6–96 h post-hatch). Larvae on moist filter paper survived for only 24 h post-hatch. Log of the mean relative expression value (REV) is plotted against each of the six time points examined. Error bars represent the pooled standard error for three biological replicates. Each biological replicate was represented by two technical replicates.

from 6 through 96 h post-hatch (in total five time-points). However, larvae reared on moist filter paper survived for only 24 h post-hatch and thus included only three of the five time-points (6, 12 and 24 h post-hatch). Quantitative analysis revealed the *MdesDAD1* mRNA to be more abundant in larvae held on moist filter paper and during incompatible interactions compared to larvae during compatible interactions (Fig. 5). The most significant ($P<0.05$) difference in the expression level of *MdesDAD1* during incompatible/compatible interactions was calculated between 48 h post-hatch larvae (Fig. 5). A 1.7-, 3.4-, and 3.2-fold increase was calculated for 12, 48, and 72 h post-hatch larvae infesting resistant wheat compared to similar aged larvae infesting susceptible wheat, respectively. However, the expression level of *MdesDAD1* transcript was observed to be non-significant ($P>0.05$; 1.3-fold) between larvae feeding on resistant/susceptible wheat plants after 96 h post-hatch (Fig. 5).

4. Discussion

Much of the existing knowledge on the expression analysis of DAD1 genes pertains to higher animals and plants. To our knowledge this is the first report of an insect DAD that could have a putative role during insect-plant interactions. In a search for genes that exhibit differential expression during Hessian fly/wheat interactions, a full length cDNA coding for a putative DAD1 was identified from an EST project focusing on the midgut of feeding Hessian fly larvae. Both blast and phylogenetic analyses confirmed the homology (annotation) of *MdesDAD1* with similar sequences from other organisms including mammals, insects and plants.

Both the tissue and the developmental expression patterns for *MdesDAD1* were assessed only in compatible Hessian fly/wheat interactions because in incompatible interactions the first-instar larvae are dead within 5–6 days after hatch (Painter, 1930; Shukle et al., 1992). The *MdesDAD1* transcript was readily detected in all the tissues examined. The expression level observed in the midgut compared to the levels in the salivary glands, fat bodies and Malphigian tubules was found to be non-significant ($P>0.05$). These results suggest an equivalent/constitutive expression in all tissues examined and collectively infer that *MdesDAD1* is expressed in all, if not most of the Hessian fly body tissues. Similar observations were reported in animals including *Mesocricetus auratus* (Nakashima et al., 1993), *A. ventricosus* (Lee et al., 2003), and plants (Gallois et al., 1997). It is unknown whether the midgut cells of the Hessian fly undergo higher apoptosis compared to other body tissues upon encountering dietary host plant allelochemicals. However, the results obtained in this study provide initial clues to the levels of apoptosis occurring in different tissues when Hessian fly larvae successfully interact with wheat (compatible interactions). Unlike the tissue expression profile of *MdesDAD1*, a clear and distinct differential expression pattern was revealed for the developmental stages examined (1st-3rd instar larvae, pupae and adults). The temporal (high) expression of *MdesDAD1* in 1st-, 2nd-, and late 3rd-instar larvae could be the result of defense against unwanted PCD stemming from ingested wheat toxins, allelochemicals. Compared to early feeding instars the later instar larvae may need to deal with greater post-ingestion stress effects (Mittapalli et al., 2007b).

In insects, the pre-pupal stage represents a dynamic transition from larvae to pupa resulting in massive tissue re-organization (developmental differentiation, Allen et al., 1991). As a result, this process could potentially provide a rich environment for PCD to occur. Thus, an alternate explanation for the peak expression of *MdesDAD1* prior to pupation (late 3rd-instar) can be attributed towards inhibition of unwanted developmentally-regulated cell death. Indeed, it was found that in *C. elegans* DAD1 expression had a defensive action against PCD during embryogenesis (Sugimoto et al., 1995).

The most intriguing expression profile for *MdesDAD1* was revealed during compatible and incompatible Hessian fly/wheat interactions. The higher expression of *MdesDAD1* observed in larvae (6–72 h post-hatch) feeding on resistant wheat compared to similar aged larvae infesting susceptible wheat suggests an anti-apoptotic role of *MdesDAD1* in larvae during incompatible interactions. The subsequent drop in *MdesDAD1* mRNA levels in older larvae (96 h post-hatch) infesting resistant wheat could indicate an early onset of the cell death pathway and thus the correlated repression of *MdesDAD1*. On the other hand, larvae participating in compatible interactions showed a low but constitute expression level for *MdesDAD1* at all the time points examined suggesting low levels of PCD in the initial phase of the interaction.

A plausible explanation for unwanted apoptotic cell death could be the result of starvation arising from stress-induced challenges. On susceptible plants, larvae establish a feeding site between 12 and 24 h post-hatch (unpublished observation, Shukle et al.) and genes involved in establishing the feeding site, manipulation of host-plant cells, feeding, and growth/development are up-regulated (Mittapalli et al., 2005a,b, 2007a). The lower level of *MdesDAD1* transcript in larvae on susceptible wheat could represent a normal part of homeostasis and development. Furthermore, a right amount of PCD associated with normal homeostasis and development could be necessary for successful development of larvae on susceptible wheat and ‘colonization’ of the wheat plant. On resistant plants

however, larvae fail to establish a feeding site and genes involved in responding to stress and disruption of homeostasis are up-regulated (Mittapalli et al., 2006, 2007b). Further, on resistant plants larvae could imbibe cell sap from individual epidermal cells and encounter toxic plant compounds, feeding deterrents that could attribute to their inability to manipulate host-plant cells to develop a nutritive tissue layer. The congruency between expression responses of larvae held on filter paper and larvae on resistant plants suggest the failure to establish a sustained feeding site ultimately leading to starvation and death.

Enhanced expression of plant resistance genes could hinder herbivore development and nutrition. In tomato plants pathogenesis-related genes (PR) were found to be up-regulated during incompatible interactions involving the potato aphid, *Macrosiphum euphorbiae* (de Ilarduya et al., 2003). Additionally, the authors also mention that aphid feeding in tomato elicits defense responses hosted by both jasmonic acid and salicylic acid defense signaling pathways and which include the action of various PR genes, lipoxygenases and proteinase inhibitors.

The results obtained in this study clearly suggest the involvement of the Hessian fly DAD1 in preventing unwanted PCD triggered by resistant factors in wheat plants. It has been reported that DAD-like genes promptly respond to external stimuli primarily temperature (Nakashima et al., 1993; Lee et al., 2003; Zhu et al., 2007). On a similar note, PCD was found to be induced by *in vitro* application of 20-hydroxyecdysone in *Bombyx mori* (Terashima et al., 2000). However, compared to animal systems, the responses of DAD1 genes to external stimuli are better studied in plants (Gallois et al., 1997; Yamada et al., 2004). All the above studies and the current report on the Hessian fly DAD1 demonstrate the anti-apoptotic potential of these genes during stress-induced challenges. Further studies could be aimed at deciphering the cytological features of PCD in Hessian fly larvae infesting different wheat plants.

Acknowledgements

The authors would like to thank the technical help provided by John Shukle and Jacob Shreve (Purdue University, undergraduate technicians). Critical comments provided by Dr. Jonathan J Neal (Department of Entomology, Purdue University) are much appreciated. This is a joint contribution of the USDA-ARS and Purdue University. This article represents the results of research only. Mention of a commercial or proprietary product does not constitute an endorsement by the USDA for its use.

References

Allen, R.G., Oberley, L.W., Elwell, J.H., Sohal, R.S., 1991. Developmental patterns in the antioxidant defenses of the housefly, *Musca domestica*. J. Cell. Physiol. 146, 270–276.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

de Ilarduya, O.M., Xie, Q.G., Kaloshian, I., 2003. Aphid-induced defense responses in Mi-1-mediated compatible and incompatible tomato interactions. Mol. Plant-Microb. Interact. 16, 699–708.

Gallois, P., Makishima, T., Hechtt, V., Despres, B., Laudie, M., Nishimoto, T., Cooke, R., 1997. An *Arabidopsis thaliana* cDNA complementing a hamster apoptosis suppressor mutant. Plant J. 11, 1325–1331.

Higuchi, Y., 2003. Chromosomal DNA fragmentation in apoptosis and necrosis induced by oxidative stress. Biochem. Pharmacol. 66, 1527–1535.

Jin, H., Liu, Y., Yang, K.Y., Kim, C.Y., Baker, B., Zhang, S., 2003. Function of a mitogen-activated protein kinase pathway in N gene-mediated resistance in tobacco. Plant J. 33, 719–731.

Jones, D.T., 1999. Protein secondary structure prediction based on position-specific scoring matrices. J. Mol. Biol. 292, 195–202.

Lee, K.S., Chung, E.H., Han, J.H., Sohn, H.D., Jin, B.R., 2003. cDNA cloning of a defender against apoptotic cell death 1 (DAD1) homologue, responsive to external temperature stimulus from the spider, *Araneus ventricosus*. Comp. Biochem. Physiol. B 135, 117–123.

Mittapalli, O., Neal, J.J., Shukle, R.H., 2005a. Molecular cloning and characterization of two digestive serine proteases from the Hessian fly, *Mayetiola destructor*. Insect Mol. Biol. 14, 309–318.

Mittapalli, O., Neal, J.J., Shukle, R.H., 2005b. Differential expression of two cytochrome P450 genes in compatible and incompatible Hessian fly/wheat interactions. Insect Biochem. Mol. Biol. 35, 981–989.

Mittapalli, O., Sardesai, N., Giovanini, M.P., Williams, C.E., Shukle, R.H., 2006. Expression patterns of antibacterial genes in the Hessian fly. J. Insect Physiol. 52, 1143–1152.

Mittapalli, O., Sardesai, N., Shukle, R.H., 2007a. cDNA cloning and transcriptional expression of a peritrophin-like gene in the Hessian fly, *Mayetiola destructor* [Say]. Arch. Insect Biochem. Physiol. 64, 19–29.

Mittapalli, O., Neal, J.J., Shukle, R.H., 2007b. Antioxidant defense response in a galling insect. Proc. Natl. Acad. Sci. U. S. A. 104, 1889–1894.

Nakashima, T., Sekiguchi, T., Kuraoka, A., Fukushima, K., Shibata, Y., Komiyama, S., Nishimoto, T., 1993. Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. Mol. Cell. Biol. 13, 6367–6374.

Orzaez, D., Granell, A., 1997. The plant homologue of the defender against apoptotic death gene is down-regulated during senescence of flower petals. FEBS Lett. 404, 275–278.

Painter, R.H., 1930. The biological strains of Hessian fly. J. Econ. Entomol. 23, 322–326.

Shukle, R.H., Grover Jr., P.B., Mocelin, G., 1992. Responses of susceptible and resistant wheat associated with Hessian fly (Diptera: Cecidomyiidae) infestation. Environ. Entomol. 21, 845–853.

Shukle, R.H., 2003. Hessian Fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). Encycl. Entomol. 1, 27–31.

Sosa, O., Gallun, R.L., 1973. Purification of races B and C of the Hessian fly by genetic manipulation. Ann. Entomol. Soc. Am. 66, 1065–1070.

Steller, H., 1995. Mechanisms and genes of cellular suicide. Science 267, 1445–1449.

Sugimoto, A., Hozak, R.R., Nakashima, T., Nishimoto, T., Rothman, J.H., 1995. dad-1, an endogenous programmed cell death suppressor in *Caenorhabditis elegans* and vertebrates. EMBO J. 14, 4434–4441.

Terashima, J., Yaswhara, N., Iwami, M., Sakurai, S., Sakurai, S., 2000. PCD triggered by insect steroid hormone, 20-hydroxyecdysone, in the anterior silk gland of the silkworm, *Bombyx mori*. Dev. Genes Evol. 11, 545–558.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The clustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 24, 4875–4882.

Tsusuki, S., Iwami, M., Sakurai, S., 2001. Ecdysteroid-inducible genes in the programmed cell death during insect metamorphosis. Insect Biochem. Mol. Biol. 31, 321–331.

Yamada, T., Takatsu, Y., Kasumi, M., Marubashi, W., Ichimura, K., 2004. A homolog of the defender against apoptotic death gene (DAD1) in senescing gladiolus petals is down-regulated prior to the onset of programmed cell death. J. Plant Physiol. 161, 1281–1283.

Yuan, J.S., Reed, A., Chen, F., Stewart, C.N., 2006. Statistical analysis of real-time PCR data. BMC Bioinformatics 7, 85–97.

Zhu, L., Song, L., Zhang, H., Zhao, J., Li, C., Xu, W., 2007. Molecular cloning and responsive expression to injury stimulus of a defender against cell death 1 (DAD1) gene from bay scallops *Agropecten irradians*. Mol. Biol. Rep. doi:10.1007/s11033-007-9061-4.